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Adenoviral-Mediated Herpes Simplex Virus Thymidine Kinase Gene Transfer: Regression of Hepatic Metastasis of Pancreatic Tumors

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Summary: Pancreatic cancer is the fifth leading cause of cancer death in the United States. Most patients have obvious metastases or locally advanced disease at the time of presentation. Surgical resection does not significantly change the clinical outcome. Combination chemotherapy induces a partial response but overall survival remains low. The aim of this study was to evaluate the feasibility of adenovirus-mediated suicide gene transduction as a therapeutic approach for pancreatic cancer. A cell line was established from a murine pancreatic ductal adenocarcinoma and intrahepatic tumors were generated by inoculation of pancreatic cancer cells into the left lateral liver lobe. Transduction efficiency was characterized in vitro and in vivo. Intrahepatic tumors were treated by intratumoral adenovirus injection in combination with intraperitoneal administration of ganciclovir. Adenovirus-mediated herpes simplex virus (HSV)–thymidine kinase (tk) gene expression followed by ganciclovir treatment was highly efficient in inhibiting pancreatic cancer cell proliferation in vitro. The proliferation of nontransduced cells was significantly reduced in the presence of HSV-tk expressing cells. Intrahepatic inoculation of pancreatic cancer cells leads to successful formation of solid adenocarcinomas in syngeneic recipients. Ad.RSV-tk injection of the tumor followed by intraperitoneal ganciclovir application caused highly significant tumor volume reduction and necrosis. These results indicate that transduction of the HSV-tk gene followed by ganciclovir is highly efficient for growth inhibition of hepatic metastases of pancreatic carcinoma. **Key Words:** Pancreatic cancer—Hepatic metastasis—Adenovirus—Thymidine kinase—Suicide gene therapy—Mice.

Pancreatic adenocarcinoma is the fifth leading cause of cancer death in the United States, with 27,000 new cases and 25,000 cancer-related deaths per year in the United States (1). At the time of diagnosis patients with this relentlessly progressive and fatal disease usually have locally advanced or metastatic disease (2). The most frequent sites of metastases are the lymph nodes (50–70%), liver (50%), lungs (20%), and peritoneum (25%) (3). Overall 5-year survival is <1% and reflects the advanced stage of disease at first presentation and lack of effective therapy. According to a survey of ~1,300 patients with pancreatic cancer presenting to the Mayo Clinic during a 2-year period, only 13% were able to undergo a resection for cure (4). Single-agent chemotherapy does not significantly improve survival for patients with nonresectable tumors (5) and the combination of 5-fluorouracil, doxorubicin (Adriamycin), and mitomycin (Mitomycin C) does not significantly improve long-term survival over 5-fluorouracil alone (6). Several studies using *cis*-platin, epirubicin, and ifosfamide have not provided a significant improvement in long-term survival either. Radiation therapy provides effective palliation in the instances of severe retroperitoneal pain due to metastasis, but this therapy is not curative. Considering the very limited options in current treatment regimens of pancreatic can-

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cer, the development of new treatment modalities, possibly in combination with current modalities, is of major importance.

Recent progress in vector development has made gene therapy a promising approach to cancer therapy. Transduced genes may be used to enhance tumor immunogenicity and the antitumoral immune response, to block activated oncogene expression, to express tumor suppressor genes, to protect normal stem cells from high-dose chemotherapy, or to introduce "suicide" genes into tumor cells (7). Suicide genes endow transduced tumor cells with enzymatic activities that allow the tumor cells to metabolize non- or minimally toxic prodrugs into highly toxic substrates that result in tumor cell killing (8–11). The most widely used suicide gene approach utilizes the herpes simplex virus–thymidine kinase (HSV-tk) to convert the prodrug ganciclovir (GCV) into its toxic phosphate. The phosphorylated compound is incorporated into newly synthesized DNA and induces chain termination, thereby selectively killing dividing cells (12). Cytotoxic sensitivity to nucleoside analogues by the introduction of HSV-tk has been demonstrated in different tumor cell lines (13–19). Direct transduction of every cell is not a prerequisite of HSV-tk-mediated cell killing. Described as the "bystander effect," transduced tumor cells can transfer the toxic metabolites to neighboring untransduced cells via gap junctions, resulting in the killing of the entire tumor population (20,21).

Initial approaches using HSV-tk utilized retroviral vector systems, which also harbor the neomycin resistance gene (22,23), for ex vivo transduction and inoculation of tumor cells into the host after selection in G418 medium. Transduction efficiency can be dramatically enhanced using adenoviruses for transduction in vivo. In the present study we have evaluated the potential of suicide gene therapy for pancreatic cancer. Transduction efficiency and suicide gene-mediated GCV sensitivity have been established in vitro. We have developed a murine, intrahepatic tumor model, simulating pancreatic cancer metastasis. Intratumoral injection of replication-deficient adenovirus expressing HSV-tk followed by GCV administration induces tumor necrosis and a highly significant tumor volume reduction.

MATERIALS AND METHODS

Recombinant adenoviruses

A replication-deficient adenovirus, expressing the HSV Type I-tk gene under the transcriptional control of the Rous sarcoma virus (RSV) long terminal repeat promoter, Ad.RSV-tk, was prepared as described previously (13). Recombinant adenovirus was amplified from a single plaque after coprecipitation of the plasmids pBHG10 and pAd.RSV-tk in 293 cells and purified using discontinuous cesium gradient ultracentrifugation. The titer of infectious particles was determined as plaque forming units (p.f.u.) utilizing a plaque assay in 293 cells. The replication-deficient adenovirus, expressing β-galactosidase (24) under the control of RSV long terminal repeat promoter (Ad.RSV-βGal), as well as the replication-deficient control virus Ad.DL312, containing an empty expression cassette, was prepared as described.

Pancreatic cancer cell line

The murine pancreatic cancer PANC02 was established in 1984 in female C57BL6 mice as described previously (25). After 3-methylcholantrene induction a ductal pancreatic adenocarcinoma was isolated and subcutaneously passaged in the syngeneic host (25). Subcutaneous tumor tissue was received from the National Cancer Institute, Developmental Therapeutics Program, Code C5702/A/67, and a pancreatic cancer cell line, required for intrahepatic tumor cell inoculation, was isolated by repeated selective trypsination to eradicate fibroblast contamination. This PANC02 cell line was maintained in minimal essential medium (MEM) cell culture medium, supplemented with 20% fetal calf serum, 2 m*M* glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were passaged by brief trypsination with 0.025% trypsin. Cell culture reagents were obtained from GIBCO.

Transduction in vitro

We evaluated the transduction efficiency of the recombinant adenovirus in PANC02 cells in vitro using the replication-deficient adenovirus Ad.RSV-βGal, a gift from M. Perricaudet, expressing the bacterial β-galactosidase gene. The backbone and promoter are similar to the adenoviral vector Ad.-RSV-tk.

One hundred thousand cells in 2 ml of complete medium were seeded in six-well tissue culture plates and incubated at 37°C and 5% CO_2 for 6 h. After washing with phosphate-buffered saline (PBS) the pancreatic cancer cells were incubated with Ad.RSV-βGal at different multiplicities of infection (m.o.i.), ranging from 0 to 3,000, in duplicate in serum-free medium. The adenovirus-containing medium was re-

placed by growth medium after 1 h of incubation at 37°C. Forty-eight hours later cells were washed twice with ice-cold PBS and fixed with PBS containing 0.5% glutaraldehyde. β-Galactosidase activity was determined by adding 2 ml of staining solution containing 1.3 m*M* $MgCl_2$, 3 m*M* $NaCl_2$, 3 m*M* potassium ferricyanide, 3 m*M* potassium ferrocyanide, 0.5 mg/ml 5-bromo-4-chlor-3-indolyl-β-galactoside (X-gal), and 44 m*M* HEPES, pH 7.3. After transduction, β-galactosidase activity occurs in the nucleus, due to a nuclear translocation signal, and transduction efficiency was quantified by counting the percentage of transduced cells identified by X-gal nuclear staining (24). ED_{50} and ED_{95} describe the m.o.i. of Ad.RSV-βGal required for 50 and 95% visible transgene expression according to this protocol.

Ad.RSV-tk transduction and tk-mediated GCV toxicity in vitro

GCV toxicity of adenovirus vector-mediated HSV-tk-transduced pancreatic cancer cells was determined utilizing a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay (26–28). Seventy-five microliters of a PANC02 cell suspension in growth medium containing 2×10^4 cells per ml was seeded into 96-well plates and incubated in complete medium. After 6 h of incubation, Ad.RSV-tk was added to the cells at different m.o.i. in a volume of 56 μl of serum-free medium. Twelve hours later medium was supplemented with GCV (10 μg/ml medium) or PBS and cells were incubated for an additional 48 h. Proliferation was determined by adding MTT, prepared as a 5 mg/ml stock solution in PBS and diluted 1:5 with high-glucose Dulbecco's modified Eagle medium (MEM) prior to use. After adding 37.5 μl, 96-well plates were further incubated at 37°C for 4 h. Medium was removed and the colored formazan product, transformed by enzymes active only in living cells, was quantified using a microtiter plate reader at 595 nm after dissolving in 150 μl of dimethyl sulfoxide.

Bystander effect of RSV-tk-mediated tumor cell killing in vitro

To demonstrate the effect of transduced tumor cells on the proliferation of neighboring, nontransduced tumor cells, PANC02 cells were transduced with Ad.RSV-tk at an m.o.i. of 400 for 1 h and, after repeated washing, mixed with nontransduced PANC02 cells at different ratios. Cells were then seeded in 96-well plates at the high density of 5×10^4 cells per ml to ensure the formation of gap junctions between transduced and nontransduced cells. Twelve hours after seeding 75 μl of the cell suspension per well, GCV (10 μg/ml), or PBS in 55 μl of high-glucose Dulbecco's MEM was added. Forty-eight hours later MTT was added, and cell proliferation determined as described above.

Establishment of a hepatic metastasis model in vivo

After passaging PANC02 cells in cell culture and setting up a cell line for subsequent hepatic injection, cells were inoculated after nine passages subcutaneously in syngeneic 10- to 12-week-old female C57BL6 mice for comparison with the original subcutaneous tumor tissue. No difference was found in the histological characterization and tumorigenicity. To establish a hepatic metastasis model for pancreatic cancer in syngeneic mice, PANC02 cells were resuspended in Earle's balanced salt solution (EBSS) after trypsination. Female C57BL6 mice were anesthetized using 10 mg of 2,2,2-tribromoethanol (Avertin) intraperitoneally and laparotomy was performed by upper median incision. The left lateral liver lobe was exposed, and 10^5 cells in a volume of 10 μl of EBSS slowly inoculated into the tip of the left lateral liver lobe using a 100-μl Hamilton syringe and a 30-gauge needle. Lesions were sealed using an electric coagulator to avoid tumor cell leakage into the abdomen.

Adenoviral transduction in vivo

Intrahepatic tumors were generated as described. Thirteen days after tumor cell inoculation intrahepatic tumors were injected with 10^8 p.f.u. of Ad.RSV-βGal in 70 μl of buffer containing 10 m*M* Tris, 1 m*M* $MgCl_2$, 20 μg/ml polybrene, and 10% (vol/vol) glycerol, pH 7.4, following anesthesia with Avertin according to the treatment procedures. Forty-eight hours after adenovirus administration animals were sacrificed and livers fixated for 2 h in PBS, pH 7.3, containing 1% formaldehyde and 0.2% glutaraldehyde. Tissues were thoroughly washed in PBS and incubated in a PBS solution containing 5 m*M* K-ferricyanide, 5 m*M* K-ferrocyanide, 2 m*M* magnesium chloride, and 1 mg/ml X-gal for 1–5 h at 37°C. Stained tissues were washed in PBS.

Gene therapy for hepatic metastasis of pancreatic cancer

Thirteen days after tumor cell inoculation mice were anesthesized and the intrah patic tumors exposed performing a second laparotomy. Tumors averaging 4–5 mm in diameter were then injected with

various doses of Ad.RSV-tk or the control virus Ad.DL312, diluted in 70 μl of buffer containing 10 m*M* Tris, 1 m*M* $MgCl_2$, 20 μg/ml polybrene, and 10% (vol/vol) glycerol, pH 7.4, using a Hamilton 100-μl syringe and 30-gauge needle. To avoid virus leakage, lesions were also sealed using a pinpoint electric coagulator. GCV was dissolved in PBS without calcium and magnesium and injected intraperitoneally at dosages of 10 mg per kg body weight, twice daily for 6 days. Mice were euthanized at day 26 after tumor cell inoculation by intraperitoneal injection of 25 mg of Avertin or maintained for long-term survival analysis. Tumor volumes were calculated by the formula of rotational ellipsoids after measurement of two diameters (29). Means and upper and lower errors were calculated according to the natural logs of the determined tumor volumes considering a nonnormal tumor volume distribution.

Histopathology and morphometric analysis

Livers were removed and preserved in PBS containing 10% formaldehyde. After hematoxylin–eosin staining, computerized morphometric analysis of the largest cross section using the point counting method with a computer-assisted digitizing system (Bioquant) was performed and at least 1,600 points per cross section were evaluated. The total area of viable tumor cells among the different groups was compared by analysis of variance (ANOVA).

All animal experiments conducted in this study were performed according to the National Institutes of Health guidelines for the use and care of laboratory animals.

RESULTS

In vitro gene transduction

The murine, transplantable tumor PANC02 was used to establish pancreatic cancer cell lines for in vitro characterization and intrahepatic tumor cell inoculation. X-gal staining after adenoviral transduction with Ad.RSV-βGal demonstrated an intense intranuclear staining that was dependent on the m.o.i. used to transduce PANC02 cells in vitro (Fig. 1). Fifty percent of the cell population was visibly transduced at an m.o.i. of 83 and >95% was transduced at an m.o.i. $\geq$480 (Fig. 2).

Toxicity of GCV was shown after in vitro transduction of PANC02 cells with the adenovirus Ad.RSV-tk expressing HSV-tk. Whereas cytopathic effects at an m.o.i. >3,000 resulted in 100% proliferation inhibition independent of GCV, presumably due to adenoviral toxicity, at a lower m.o.i. the presence of GCV significantly enhanced toxicity, with cytopathic effects at an m.o.i. as low as 3 for 50% proliferation inhibition (Fig. 3). At an m.o.i. of 30, cell proliferation was reduced to 10% in the presence of GCV, compared to only a minor growth inhibition in the absence of GCV.

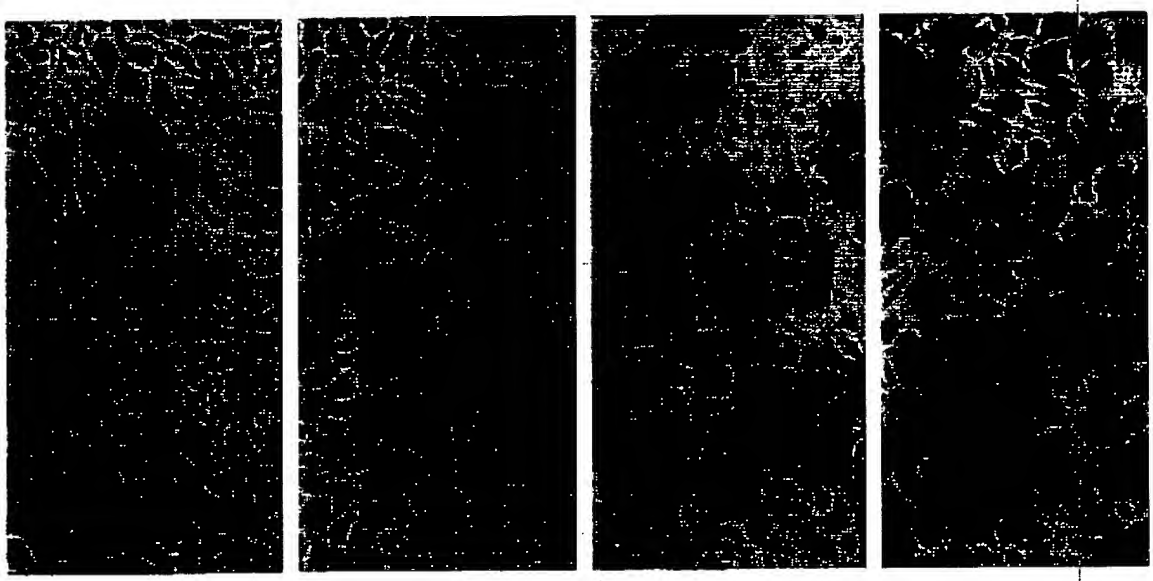

FIG. 1. In vitro transduction of PANC02 cells with Ad.RSV-βGal. After infection of PANC02 cells with various m.o.i. of Ad.RSVβGal, cells were stained with X-gal 48 h later. **A:** m.o.i. = 3; **B:** m.o.i. = 30; **C:** m.o.i. = 300; **D:** m.o.i. = 3,000. Original magnification, 40×.

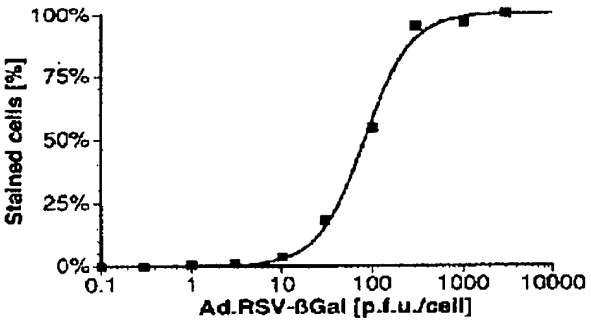

FIG. 2. Adenoviral transduction efficiency in PANC02 using Ad.RSV-βGal. At an m.o.i. of 83, 50% of the cells stain blue, and at an m.o.i. of 480, 95% of the cells have visible transgene expression.

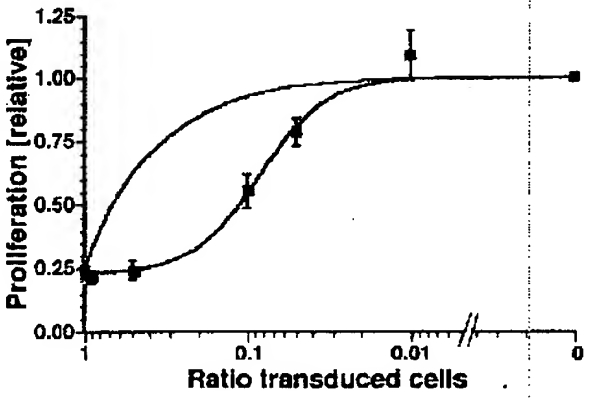

FIG. 4. In vitro demonstration of the bystander effect in PANC02 cancer cells. PANC02 cells were transduced with Ad.RSV-tk at an m.o.i. of 400 for 1 h, trypsinased, washed twice, and mixed with nontransduced cells at different ratios. After 12 h of incubation, GCV at a concentration of 10 μg/ml was added, and 48 h later cell proliferation was determined using the MTT assay. (■) Actual proliferation; (—) control proliferation without the bystander effect.

Bystander effect of tk-mediated GCV toxicity toward PANC02

The bystander effect on the inhibition of proliferation due to tk-mediated toxification of GCV was demonstrated in vitro by mixing transduced and nontransduced PANC02 cells at various ratios, following incubation in the presence of GCV. In contrast to the previous experiment, the supernatant containing the adenovirus Ad.RSV-tk was removed after 1 h of incubation and cells were washed thoroughly to avoid viral contamination when mixing with nontransduced cells. When mixed with nontransduced cells at a ratio of 1:2 (50% transduced cells), proliferation remained low, at 25% (Fig. 4). At a ratio of 1:10 (10% transduced cells) proliferation was 55% of controls, which was significantly lower than the assumed proliferation of 92.5% negotiating the inhibition of proliferation by neighboring transduced cells. The additional proliferation inhibition, described as the "bystander effect," could be demonstrated for as few as 5% transduced cells. To estimate the degree of adenoviral contamination, the titer of Ad.RSV-tk in the suspension of transduced cells was determined using a 293 cell-based plaque assay. With 50% transduced cells being used, adenoviral contamination was $\leq$0.01 m.o.i., and with 10% transduced cells adenoviral contamination was <0.002 m.o.i.

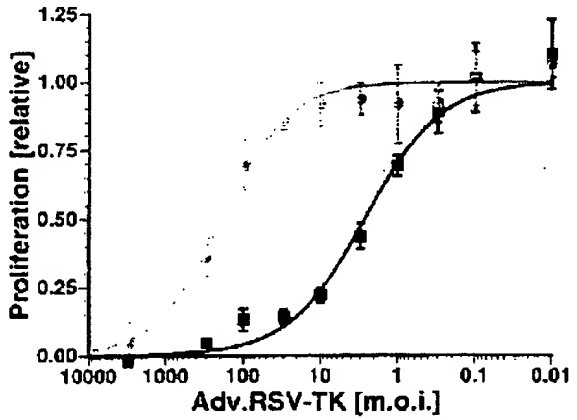

FIG. 3. In vitro determination of GCV toxicity against PANC02 cancer cells, transduced with different m.o.i. of Ad.RSV-tk in the absence (●) or presence (■) of GCV at a concentration of 10 μg/ml. Cell proliferation was quantified 48 h later by MTT staining and compared to that of nontransduced cells (100%).

Tumor model for liver metastasis of pancreatic cancer

Tumorigenicity of the PANC02 cell line was demonstrated by subcutaneous inoculation of various amounts of cancer cells and observation of subcutaneous tumor growth in the syngeneic host, 10- to 12-week-old female C57BL6 mice (Fig. 5). Inoculation of 1×10^5 cells generated tumors at a frequency of 100% ($n = 5$). Tumor growth was dose dependent of the number of cells injected. The histology of these subcutaneous tumors was then compared with the original tumor tissue obtained from the National Cancer Institute. They were indistinguishable in differentiation, necrosis, fibrotic reaction, and inflammation. Intrahepatic inoculation of PANC02 cells resulted in 80% tumor formation when 5×10^4 cells ($n = 5$) were inoculated and 100% tumor formation when 1×10^5 cells ($n = 9$) were inoculated. After 13 days mice developed intrahepatic tumors with tumor

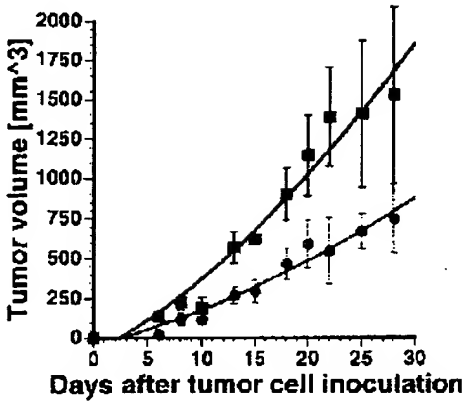

FIG. 5. Tumorigenicity of PANC02. Growth characteristics of PANC02 cells after subcutaneous inoculation of 5×10^5 (■) or 1×10^5 (●) cells in the syngeneic host was determined by measuring outside tumor diameters and calculating the tumor volume as described (29).

FIG. 6. Transduction efficiency of hepatic metastasis of pancreatic cancer using the adenovirus Ad.RSV-βGal. Established tumors were injected with 10^8 p.f.u. of Ad.RSV-βGal, and 48 h later tissues were stained with X-gal. The tumor located in the left lateral tip of the liver shows intense blue staining after 1 h of incubation in X-gal staining solution.

diameters ranging from 2.0 × 2.0 to 6.5 × 8.0 mm. The mice with tumors 4.0 × 5.0 to 5.0 × 5.5 mm were suitable for intratumoral adenovirus administration. Untreated mice died at 32 to 45 days, with an average of 34 days, after tumor cell inoculation (n = 23). Necropsy revealed multiple lung metastasis as the most probable cause of death.

Adenoviral transduction in vivo

According to the treatment conditions, 10^8 p.f.u. of Ad.RSV-βGal in a volume of 70 μl was injected in preformed hepatic tumors measuring 4–5 mm in diameter. Staining of the hepatic tissue containing the pancreatic tumor 48 h after adenoviral transduction showed high β-galactosidase activity in the tumor after 1 h of incubation (Fig. 6). Further incubation for 24 h showed weak diffuse staining of the entire liver in adenovirally transduced as well as nontransduced animals, demonstrating endogenous hepatic β-galactosidase activity. This finding illustrates the feasibility of localized intratumoral gene transduction and expression after injection of adenoviruses.

Suicide gene-mediated tumor regression in vivo

An Ad.RSV-tk dose escalation study was designed to determine the appropriate dose of adenovirus with optimal tumor killing associated with a minimal hepatotoxicity. Different doses of Ad.RSV-tk, ranging from 3×10^7 to 2×10^9 p.f.u., were injected into established intrahepatic tumors. At doses of $\geq 3 \times 10^8$ p.f.u., 75–100% of animals died during treatment. Histology revealed severe inflammation of the liver. At doses of $\leq 1 \times 10^8$ p.f.u., all animals survived the treatment procedure and histologic observation revealed only minimal periportal inflammation. All further experiments were performed using this viral dose of 1×10^8 p.f.u.. Established intrahepatic tumors were injected with (group 1) Ad.RSV-tk at 10^8 p.f.u. (n = 16), (group 2) the replication-deficient control virus Ad.DL312 at 10^8 p.f.u. (n = 5), or (group 3) PBS (n = 5). Eight mice in group 1 received 10 mg of GCV i.p. twice per day over the next 6 days (group 1A); the other eight mice received PBS with the same schedule (group 1B). All mice in group 2 received GCV i.p. twice per day over the next 6 days and all mice in group 3 received PBS. The mice in each group appeared to be in good health over the entire treatment period and were sacrificed at day 26 for macroscopic and histologic analysis of tumor regression. As shown in Figs. 5 and 6, mice in group 3 had large hepatic adenocarcinomas with moderate differentiation, minimal inflammation, and minimal focal necrosis. Control group 1B showed virtually no difference in tumor size or necrosis compared to group 3. Mice injected with the control adenovirus Ad.DL312 and GCV showed a slight, although not significant reduction in tumor volume compared to the PBS control group. Mice in treatment group 1A, receiving Ad.RSV-tk and GCV, showed a dramatic reduction in tumor volume (Figs. 7 and 8) ($p \leq 0.0001$) compared to the PBS control group. Histologic study of intrahepatic carcinomas treated with Ad.RSV-tk and GCV showed large necrotic areas. At the border with the hepatic parenchyma, infiltrating viable tumor cells were surrounded by an intense

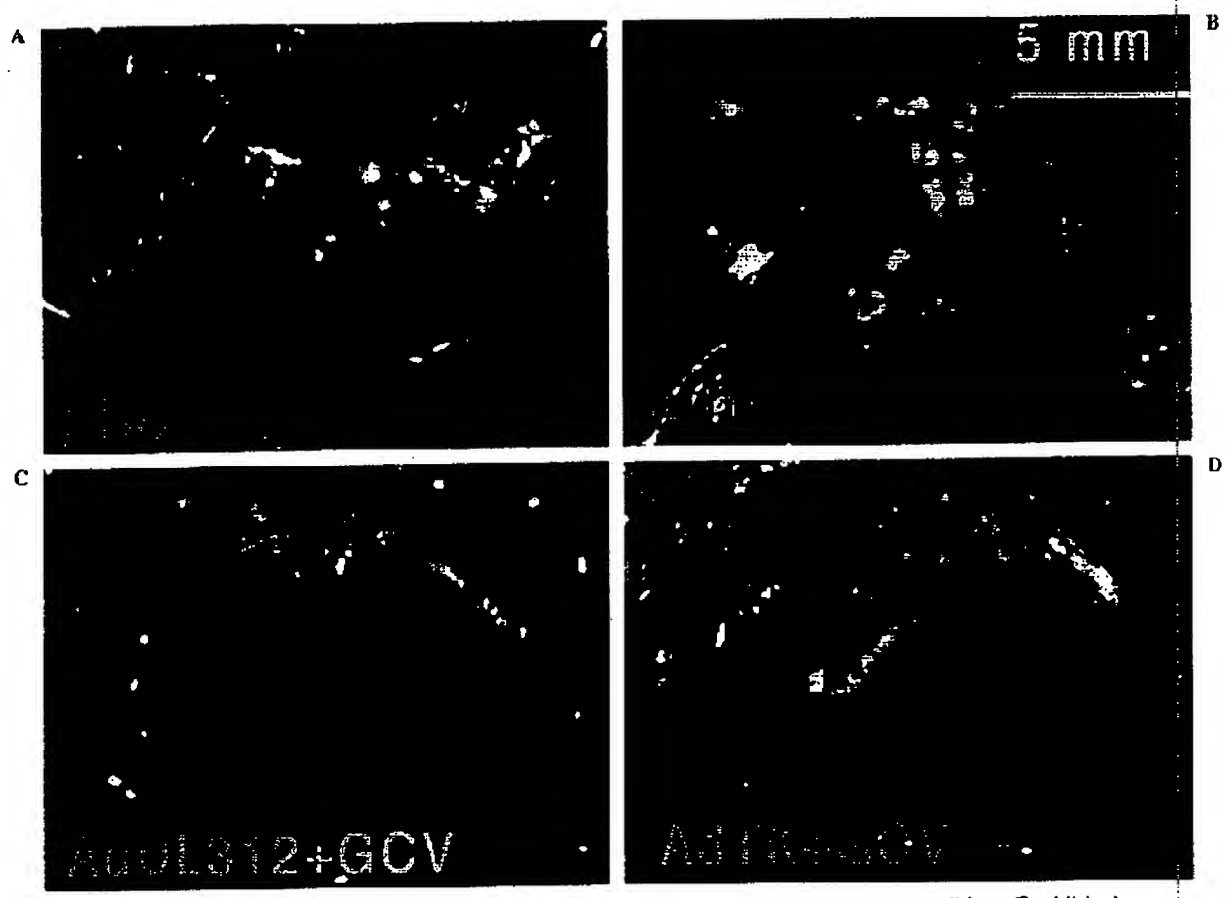

FIG. 7. Macroscopic evaluation of hepatic metastasis of pancreatic cancer after different treatment conditions. Established tumors were injected with either Ad.RSV-tk, Ad.DL312, or PBS and mice subsequently treated with GCV or PBS i.p. for the following 6 days. All mice were sacrificed at day 26. Tumors in the treatment group Ad.RSV-tk + ganciclovir show a dramatic reduction in tumor volume over all other control groups.

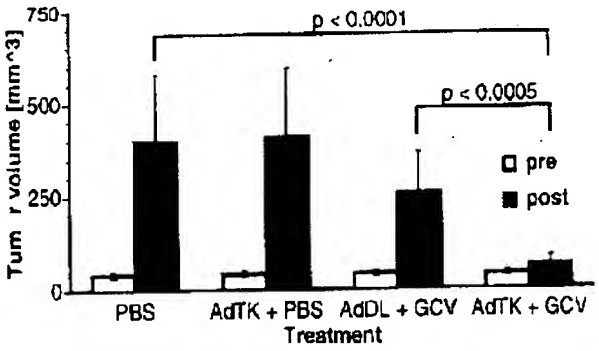

FIG. 8. Tumor regression of hepatic metastasis of pancreatic cancer. Differences in tumor volumes: AdTK + GCV versus AdDL + GCV, $p < 0.0005$; AdTK + GCV versus AdTK + PBS, $p < 0.0001$; AdTK + GCV versus PBS, $p < 0.0001$. No significant difference between control groups: PBS versus AdTK + PBS, $p = 0.87$; PBS versus AdDL + GCV, $p = 0.0884$.

inflammatory reaction with neutrophilic and lymphocytic infiltration (Fig. 9). Tumors injected with the control adenovirus Ad.DL312 followed by treatment with GCV exhibited moderate differentiation and a high mitotic rate with minimal neutrophilic infiltration, focal necrosis, moderate fibrotic reaction, and many tumor cells infiltrating the surrounding hepatic tissue. Computerized morphometric analysis confirmed but did not further enhance the significant difference of tumor volumes in the tk-GCV group versus controls because these tumor cells generate an inflammatory and strong desmoplastic reaction (Fig. 9) and those remain, even when the treatment has successfully reduced the total tumor volume.

DISCUSSION

Due to the late development of clinical symptoms, pancreatic carcinoma is usually diagnosed at a late

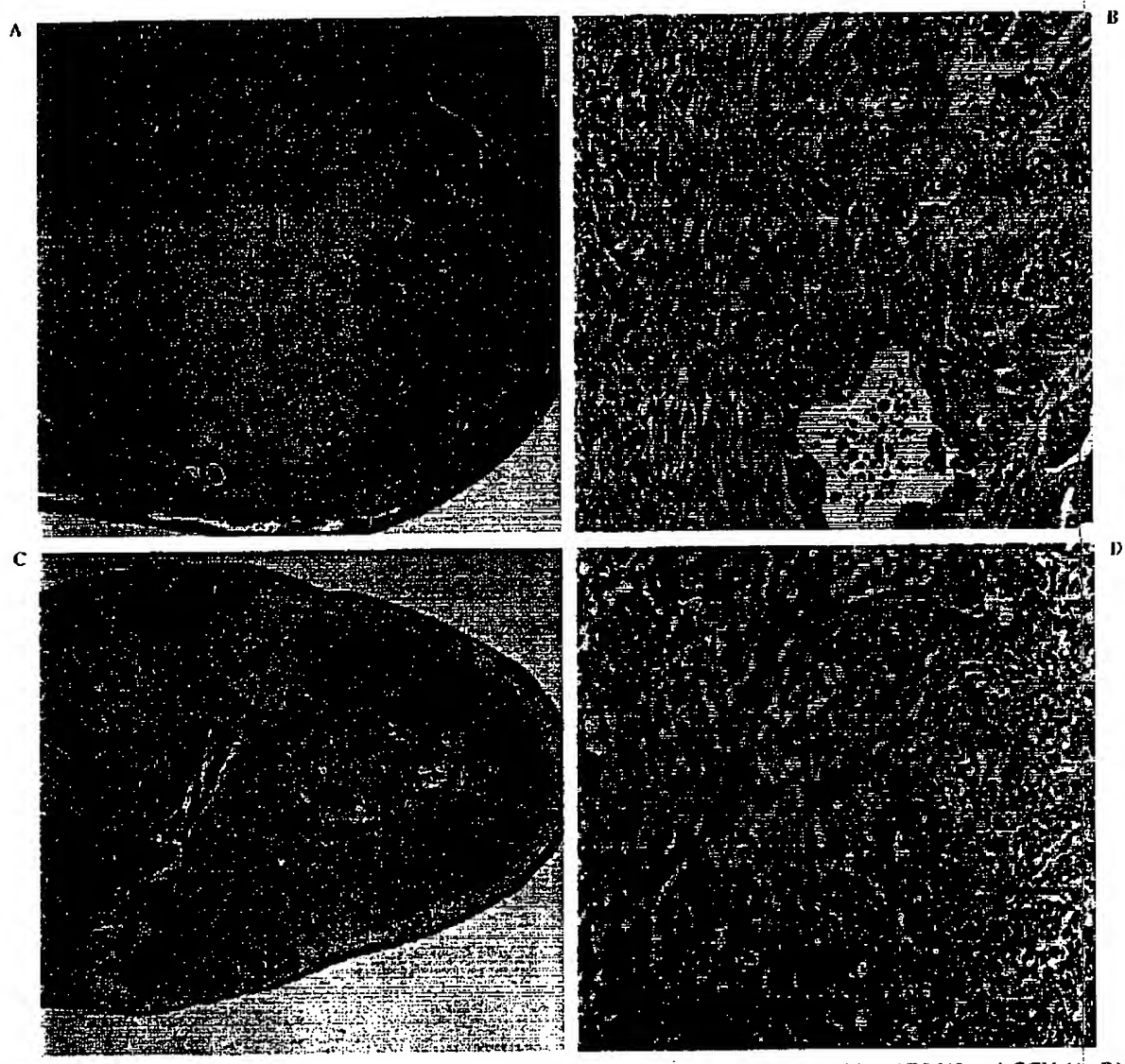

FIG. 9. Hematoxylin–eosin staining of hepatic metastasis of pancreatic cancer after treatment with Ad.DL312 and GCV (**A, B**) or Ad.RSV-tk and GCV (**C, D**) at 25× to demonstrate tumor regression and necrosis. A 200× magnification shows the intense inflammatory infiltrate at the junction of the carcinoma and the liver (**left side**) in the TK + GCV treatment-only group. Lesser degrees of inflammation were found in the controls. The mitotic activity (frequency of mitotic cell figures) among the adenocarcinoma cells is very high in the control groups compared to the TK + GCV group.

stage, with tumor infiltration of neighboring organs or metastasis. Therapeutic options are limited and life expectancy remains dismal. Therefore it is of major importance to develop new therapeutic strategies. Gene therapy approaches, targeting only the tumor cell population and therefore being more specific than systemic chemotherapy, may result in an improved therapeutic index with reduced systemic toxicity. The use of suicide genes such as HSV-tk has been evaluated in different tumor models (13–19). Prior efforts using retroviruses required transduction of tumor cells in vitro (15,16) or intratumoral inoculation of packaging cells (12). Compared to retroviruses, recombinant adenoviruses have high viral titers, high transduction efficiencies in vivo, and apparent safety in clinical trials (30). Considering the application of adenovirus-mediated suicide gene therapy to pancreatic cancer, we evaluated the effi-

cacy of Ad.RSV-tk in a syngeneic murine model. The syngeneic murine pancreatic cancer model was preferred to a nude mouse model, because the immunocompetence of the host is considered to have an impact on the efficiency of HSV-tk and GCV treatment (31).

A high transduction efficiency was demonstrated in vitro using the adenovirus Ad.RSV-βGal and subsequent X-Gal staining, with a resulting ED_{50} of 83 m.o.i. and ED_{95} of 480. Transduction with Ad.RSV-tk rendered pancreatic cancer cells highly sensitive to GCV, with 50% inhibition of proliferation at an m.o.i. of 3 and 90% inhibition at an m.o.i. of 30 in the presence of GCV. In the absence of GCV, 100-fold higher adenovirus concentrations are required to obtain the same effect on proliferation inhibition, due mainly to the basic adenoviral toxicity. At an m.o.i. of 3,000 proliferation is reduced to <10% of controls, with transgene expression at this m.o.i. demonstrated to be 100% using Ad.RSV-βGal.

The bystander effect (21,32) describes the proliferation inhibition of nontransduced cells, caused by the transport of toxic metabolites via gap junctions from neighboring transduced cells. We demonstrated the bystander effect for HSV-tk-mediated GCV toxicity in this pancreatic cancer cell line. Mixing of 5% transduced cells with 95% nontransduced cells resulted in 75% proliferation compared to nontransduced cells. With only 10% transduced cells proliferation of the entire cell population was reduced to 50% over controls. The remaining proliferation of transduced cells can be explained by adenoviral incubation at an m.o.i. of 400 limited to 1 h, followed by extensive washing to avoid adenoviral contamination of the nontransduced cell portion. To exclude possible adenoviral transduction of the nontransduced cell portion when mixed with transduced cells, adenoviral contamination of the transduced cell suspension was determined using a plaque assay. Adenoviral contamination was <0.01 m.o.i. with no inhibition of PANC02 cell proliferation in the presence of GCV (Fig. 3). Therefore the effect of adenoviral contamination is negligible.

Transduction efficiency, sensitivity to GCV after HSV-tk transduction, and the bystander effect made an approach promising to reduce significantly preformed intrahepatic PANC02 tumors.

A murine model of hepatic metastasis of pancreatic cancer was established. The more complex hepatic tumor model rather than a subcutaneous model was used for two reasons: it more closely resembles the clinical problem in humans and it permits simultaneous evaluation of treatment-related hepatotoxicity, which could significantly reduce the treatment benefit. In fact, the dose of Ad.RSV-tk in combination with GCV administered to intrahepatic PANC02 tumors was limited to 1×10^8 p.f.u. due to severe hepatotoxicity and treatment-related mortality at 3- to 10-fold higher doses. In our hands, intrahepatic tumor cell injection generates tumors of relatively predictable growth rate and lethality that are accessible for measurement and tumor volume quantification. We demonstrated highly significant regression of established hepatic pancreatic tumors transduced with nonlethal doses of Ad.RSV-tk in vivo and subsequently treated with GCV over all control groups. Tumor regression was determined by a striking reduction in tumor volume secondary to treatment-induced necrosis. No hepatotoxicity or tumor growth inhibition was found using the adenoviral vector alone. GCV in the absence of Ad.RSV-tk caused a slight, although not statistically significant tumor volume reduction, possibly due to endogenous intratumoral tk expression (33,34). The results of this study illustrate the feasibility of suicide gene therapy as a potential adjuvant treatment for metastatic pancreatic cancer. While suicide gene therapy is very effective in intrahepatic tumor debulking, long-term survival requires enhancement of the systemic immune response. A combination of this suicide gene approach with local cytokine expression (35–37) is promising for the further investigation of therapeutic approaches to metastatic pancreatic cancer.

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REFERENCES

1. Gold EB. Epidemiology of and risk factors for pancreatic cancer. *Surg Clin North Am* 1995;75:819–43.
2. Ettinghausen SE, Schwartzentruber DJ, Sindelar WF. Evolving strategies for the treatment of adenocarcinoma of the pancreas. *J Clin Gastroenterol* 1995;21:48–60.
3. DiMagno EP. Pancreatic adenocarcinoma. In: Yamada, Alpers, Owyang, Powell, Silverstein, eds. *Textbook of gastroenterology*. Philadelphia: Lippincott, 1995:2113–31.
4. Dalton RR, Sarr MG, van Heerden JA, Colby TV. Carcinoma of the body and tail of the pancreas: is curative resection justified? *Surgery* 1992;111:489–94.
5. Schein PS, Lavin PT, Moertel CG, et al. Randomized phase II clinical trial of adriamycin, methotrexate, and actinomycin D in advanced measurable pancreatic carcinoma. *Cancer* 1987;42:19.
6. Cullinan SA, Moertel CG, Fleming TR, et al. A comparison of three chemotherapeutic regimens in the treatment of advanced pancreatic and gastric carcinoma. *JAMA* 1985;253:2061.

7. Culver KW, Blaese RM. Gene therapy for cancer. *Trends Genet* 1994;10:174–8.
8. Moolten FL. Drug sensitivity ("suicide") genes for selective cancer chemotherapy. *Cancer Gene Ther* 1994;1:279–87.
9. Blaese RM, Ishii-Morita H, Mullen C, et al. In situ delivery of suicide genes for cancer treatment. *Eur J Cancer 30a* 1994;8:1190–3.
10. Mullen CA. Metabolic suicide genes in gene therapy. *Pharmacol Ther* 1994;63:199–207.
11. Connors TA. The choice of prodrugs for gene directed enzyme prodrug therapy of cancer. *Gene Ther* 1995;2:702–9.
12. Caruso M, Panis Y, Gagandeep S, Houssin D, Salzmann JL, Klatzmann D. Regression of established macroscopic liver metastases after in situ transduction of a suicide gene. *Proc Natl Acad Sci USA* 1993;90:7024–8.
13. Chen SH, Shine HD, Goodman JC, Grossman RG, Woo SL. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer in vivo. *Proc Natl Acad Sci USA* 1994;91:3054–7.
14. Chen SH, Chen XH, Wang Y, et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. *Proc Natl Acad Sci USA* 1995;92:2577–81.
15. Culver KW, Van Gilder J, Link CJ, et al. Gene therapy for the treatment of malignant brain tumors with in vivo tumor transduction with the herpes simplex thymidine kinase gene/ganciclovir system. *Hum Gene Ther* 1994;5:343–79.
16. Huber BE, Richards CA, Krenitsky TA. Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: an innovative approach for cancer therapy. *Proc Natl Acad Sci USA* 1991;88:8039–43.
17. O'Malley BWJ, Chen SH, Schwartz MR, Woo SL. Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. *Cancer Res* 1995; 55:1080–5.
18. Perez-Cruet MJ, Trask TW, Chen SH, et al. Adenovirus-mediated gene therapy of experimental gliomas. *J Neurosci Res* 1994;39:506–11.
19. Bonnekoh B, Greenhalgh DA, Bundman DS, et al. Inhibition of melanoma growth by adenoviral-mediated HSV thymidine kinase gene transfer in vivo. *J Invest Dermatol* 1995;104:313–7.
20. Wu JK, Cano WG, Meylaerts SA, Qi P, Vrionis F, Cherington V. Bystander tumoricidal effect in the treatment of experimental brain tumors. *Neurosurgery* 1994;35:1094–102, 1102–3.
21. Bi WL, Parysek LM, Warnick R, Stambrook PJ. In vitro evidence that metabolic cooperation is responsible for the bystander effect observed with HSV tk retroviral gene therapy. *Hum Gene Ther* 1993;4:725–31.
22. Uckert W, Walther W. Retrovirus-mediated gene transfer in cancer therapy. *Pharmacol Ther* 1994;63:323–47.
23. DiMaio JM, Clary BM, Via DF, Coveney E, Pappas TN, Lyerly HK. Directed enzyme pro-drug gene therapy for pancreatic cancer in vivo. *Surgery* 1994;116:205–13.
24. Jaffe HA, Danel C, Longenecker G, et al. Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat Genet* 1992;1:372–8.
25. Corbett TH, Roberts BJ, Leopold WR, et al. Induction and chemotherapeutic response of two transplantable ductal adenocarcinomas of the pancreas in C57BL/6 mice. *Cancer Res* 1984;44:717–26.
26. Cole SP. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemother Pharmacol* 1986;17:259–63.
27. Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* 1987;56:279–85.
28. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987;47:936–42.
29. Janik P, Briand P, Hartmann N. The effect of estrone progesterone treatment on cell proliferation kinetics of hormone dependent GR mouse mammary tumors. *Cancer Res* 1975; 35:3698–704.
30. Crystal RG, McElvaney NG, Rosenfeld MA, et al. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nature Genet* 1994;8:42–51.
31. Vile RG, Nelson JA, Castleden S, Chong H, Hart IR. Systemic gene therapy of murine melanoma using tissue specific expression of the HSVtk gene involves an immune component. *Cancer Res* 1994;54:6228–34.
32. Elshami AA, et al. Gap junctions play a role in the "bystander effect" of the herpes simplex virus thymidine kinase/ganciclovir system in vitro. *Gene Ther* 1996;3:85–92.
33. Bradshaw HD Jr. Molecular cloning and cell cycle-specific regulation of a functional human thymidine kinase gene. *Proc Natl Acad Sci USA* 1983;80:5588–91.
34. Bradshaw HD Jr, Deininger PL. Human thymidine kinase gene: molecular cloning and nucleotide sequence of a cDNA expressible in mammalian cells. *Mol Cell Biol* 1984;4:2316–20.
35. Addison CL, Braciak T, Ralston R, Muller WJ, Gauldie J, Graham FL. Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model. *Proc Natl Acad Sci USA* 1995;92:8522–6.
36. Gansbacher B. Cytokine gene therapy. *Mt Sinai J Med* 1994;61:301–9.
37. Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol* 1995;13:399–415.